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(54) Enhancement of plant metabolite production by timed elicitation.

(57) Methods are provided for maximizing the production of secondary metabolites produced from plant cell cultures. The methods involve monitoring the physiological state of the culture cells so that elicitors, which stimulate metabolite production, can be applied at an optimum time. This enhanced production of metabolites following elicitation can be further maximized by the addition of cell viability stabilizers and/or nutrients at the time of elicitation.

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MAXIMIZED ENHANCEMENT OF PLANT METABOLITE PRODUCTION BY TIMED ELICITATION

This invention relates to the production of metabolites from cell cultures.

Plants are increasingly important sources of chemicals used in medicine, fruit flavors and aromas, cosmetics and agrochemicals. Most of these chemicals are secondary metabolites such as phenolic compounds and alkaloids. Interest in these compounds has heightened as their beneficial effects are proven. For example, flavones have been shown to participate in binding to biopolymers (enzymes) and cell membranes, complexing of heavy metal ions, and electron transfer in enzyme systems, e.g. oxidoreductases, and free radical scavenging. Flavones also possess marked antiallergic activity. These metabolites have also demonstrated antihaemorrhagic, circulation-promoting, antiphlogistic, and cardiovascular activities.

Most of these chemicals are extracted directly from plant tissues. However, there is increasing interest in producing these metabolites in plant cell culture. The advantages of cell culture over the conventional agricultural production of plant material are numerous. The production of botanical products would be possible independent of environmental factors, such as climate, plant pests, and seasons, and it would be possible to produce them at any place on earth. Also, the quality of the raw products could be produced at a uniformity never observed with field grown material. Further, any newly discovered beneficial plants could be put immediately into culture circumventing the need for lengthy agricultural propagation and cultivation. However, the criterion which governs utilization of these techniques in industry is economic. Only if the product under consideration can be produced industrially by cell culture techniques at a cost equal to or lower than field-produced goods will the biotechnological realization of the cell culture method advance.

Plant cells cannot compete with true microorganisms, like bacterial or fungal cells, for the production of primary metabolites: growth and metabolic rates of plant cells are too slow compared to these organisms. The opportunity for an industrial application of plant cell culture will almost exclusively lie in the production of the specific and valuable low or high molecular weight products which are synthesized only by higher plants. For the most part, the potential for the chemical synthesis of these so-called secondary compounds is not realized.

In order to realize the industrial application of plant cell culture for the production of useful secondary metabolites, it is essential to enhance the rates of biosynthesis. Towards this goal, selection mechanisms have been utilized to identify cell lines with improved biosynthetic capabilities. These high yielding strains must be stable to be useful for the industrial production of metabolites by the cell culture system. Further, factors which influence the growth and product formation of cell cultures such as natural or synthetic plant hormones or effectors, have to be considered.

Attempts to increase metabolite production have been attempted by modifying culture conditions. One such method to increase secondary metabolites calls for culturing plant cells, first in a liquid medium suited to the growth of the cells, with a subsequent culturing step in modified liquid medium suited to the production of secondary metabolites. This method is both time consuming and laborious. The present invention is drawn to methods for the maximization of metabolite production from cell cultures.

Relevant Literature

U.S. Patent, 4,717,664, discloses a method for producing secondary metabolites of higher plants using suspension cultures of undifferentiated cells. The cultures are carried out in at least two stages in liquid media. The article "Rapid transient induction of phenylalanine ammonia-lyase mRNA in elicitor-treated bean cells" by Edwards, et al, Proc. Nat. Acad. of Sci., USA (1985) 82:6731-6735 discloses that elicitor-treated cells of Phaseolus vulgaris show a rapid stimulation of phenylalanine ammonia-lyase mRNA synthesis as an early event in the defense response leading to accumulation of phenylpropanoid-derived phytoalexins. The article "Phytoalexin synthesis in soybean cells: elicitor induction of phenylalanine ammonia-lyase and chalcone synthase mRNAs and correlation with phytoalexin accumulation" by Ebel, et al in Archives of biochemistry and biophysics (1984) 232: 240-248 discloses that fungal elicitors induce large and rapid increases in the activities of enzymes of general phenylpropanoid metabolism, phenylalanine ammonia-lyase, and of the flavonoid pathway, acetyl-CoA carboxylase and chalcone synthase in suspension cultured soybean cells.

SUMMARY OF THE INVENTION

Methods for maximizing the levels of secondary metabolites produced from cell cultures are provided. The methods involve monitoring the physiological state of the culture cells so that elicitors, which stimulate metabolite production, can be applied at an optimum time. Enzyme assays for extracellular products can be used to indicate the optimum time for the addition of elicitors. This enhanced production of secondary metabolites following elicitation can be further maximized by the addition of cell viability stabilizers and/or nutrients at the time of elicitation.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the present invention, methods are provided for maximizing the production of secondary metabolites from cell cultures. Elicitors, which stimulate metabolite production, are utilized to produce economically viable levels of specific products by maximizing their production at an optimal time in the growth phase of a plant cell suspension culture. By carefully timing the addition of elicitors, secondary metabolite production is enhanced and thus, increased metabolite yield by cells can be achieved in significantly shortened periods of time, as compared to normal growth conditions. For example, the instant Experimental section demonstrates that secondary metabolite production in a 48 hour period can be, at least, doubled by the addition of an elicitor to the culture medium at an optimum time, from about 67.4 mg/l to at least 137.3 mg/l (Table 2). Further, by adding cell viability stabilizers and/or nutrients at the time of elicitation the increased metabolite production can be stabilized rendering the metabolite production system more efficient and economic. The addition of elicitors induces large and rapid increases in the activities of enzymes of general phenylpropanoid metabolism and of the flavonoid pathway. This sudden increase in metabolism can have inhibitory or deleterious effects on the plant cells. The addition of cell viability stabilizers and/or nutrients at the time of elicitation function to maintain and extend cell viability following product formation after elicitation.

Cell cultures of the present invention are plant cell cultures. Cells from any plant species which are capable of growth or proliferation in culture may be used. The particular plant culture will be chosen based upon the metabolite of interest. Thus, cell cultures which are suitable for use include any which produce the metabolite of interest and respond to elicitation. Species of plants which may be utilized are not limited, and may include, among others, plants from Juglandaceae, Boraginaceae, Pyrolaceae, Plumbaginaceae, Lytharaceae, Solanaceae, and Ranunculaceae.

The culture medium utilized is any which is suited to the growth and proliferation of cells including Linsmaier-Skoog medium, Murashigae-Skoog medium, Gamborg et al.'s B-5 medium and the like. It is recognized that a wide choice of media is available and many modifications in the culture medium are possible. Changes in hormones, vitamins or other substituents or their concentrations are examples of possible variations.

For the most part, the plant cells will be callus, gall, or undifferentiated cells. Several methods exist for obtaining undifferentiated cells. In general, tissue samples from a plant part such as roots, leaves, stems, seeds, or other parts are sterilized and added to a solid medium where a part of the tissue proliferates into a mass of undifferentiated cells or callus. These undifferentiated cells provide an inoculant for suspension cultures. The present invention can be used with any of the wide variations which are possible in cell culture work; in the types of cells used, the tissue from which the cells are derived and the medium used to grow the cells.

Selected plant cells can be grown by any of the fermentation techniques known for plant cells. Various reactor systems include the airlift reactor, shake flask, turbine impellers, draft tube with Kaplan turbine and the like. Because of their size, plant cells are sensitive to shear stress and special care is needed with highly viscous fermentation. Additional care must also be taken for sterility of the system prior to the onset of elicitation

Plant cells can be grown in uniform well-defined sterile medium on a large scale. Large populations of plant cells can therefore be obtained rapidly in a relatively uniform state. Plant cells in culture do not necessarily exhibit any of the patterns of development observed for cells in intact whole plants. Rather, plant cells in culture are in a unique developmental state. For example, different isozymes of aspartate kinase are produced by carrot cells during different phases of culture growth (Davies et al. Plant Physiol. (1978) 61, Abstr. 526) and variations in the expression of peroxidase isozymes have been noted for cells in different clump sizes (Verma et al. Can. J. Biochem. (1970) 48:444-449). The present invention recognizes the differences in metabolic activities such that elicitation of secondary metabolites occurs at the optimum time in the culture cycle.

The physiological state of the culture is monitored to determine the optimum time for the addition of elicitors. By physiological state is intended the phase of growth of the cell culture, i.e. stationary, exponential, etc. The optimum time for the addition of elicitors to the culture is approximately the end of the exponential growth phase. That is, the optimum time is the time in which the cells have optimum enzyme potential as well as cell viability for responding to elicitors. Methods to determine the optimum time for the addition of elicitors includes measuring biomass production, measuring carbohydrate utilization, and assaying for extracellular products. The production of biomass or cell dry weight, is a good indicator of cell growth. A constant biomass for a period of about 1 day generally indicates that cells have reached approximately the end of the exponential growth phase and have entered the stationary growth phase. Thus, about the end of exponential growth phase is defined as the period when the culture biomass is constant for a time of about 12 hours to about 2 days usually about 1 day. In this manner, direct measurements of biomass can be performed to determine the growth phase of the culure.

A declining growth rate is also reflected in a declining rate of carbohydrate utilization. Thus, monitoring levels of carbohydrate utilization could also be used to determine the optimum time for the addition of elicitors.

However, it has been determined that assays for extracellular products which parallel cell growth, such as extracellular peroxidase activity, provide alternative means to determine the physiological growth phase of cultures. Assaying for extracellular peroxidase activity provides a particularly efficient, sensitive and economical means to measure biomass, i.e., culture growth. Extracellular peroxidase activity closely parallels growth in cell cultures as demonstrated in Arachis hypogea, Nicotiana tobaccum and Glycine max. Thus, the correlation between cell dry weight and extracellular peroxidase activity provides a novel alternative means to measure biomass production indirectly by measuring peroxidase activity.

The present method calls for maximizing levels of metabolites from cell cultures. By maximizing is intended, the production of a nigher concentration of metabolites in a shortened period of time as compared to normal growth conditions. That is, the time during which the optimum quantity of secondary metabolites is produced is reduced by the addition of elicitors into the cell cultures at an optimum time. Phenolic yields in the range of about 0.12 g/l to about 0.15 g/l can be realized using elicitation. Even greater enhancement of metabolite production can be attained by a combination of timed elicitation, addition of cell viability stabilizers and/or the addition of nutrients at the time of elicitation.

Elicitation is an induction process which results in increases in the levels of mRNA for phenylalanine ammonia-lyase (PAL), chalcone synthase, chalcone isomerase, etc. being the first responses to elicitor contact in a cascade of biochemical events. This induction process includes increased activities of enzymes of general phenylpropanoid metabolism, of flavonoid biosynthesis, of pterocarpan biosynthesis, of defense response, etc. Included within the induction process triggered by elicitors is the synthesis of low molecular weight secondary metabolites, i.e., aromatic compounds of about 100 to about 1,000 daltons (d). The maximum yield of metabolites which result from elicitation is generally found after about 10 hours to about 5 days of exposure to an elicitor. For purposes of the present invention the time for maximum yield of metabolites will vary for each cell culture, but can readily be determined by a time course experiment. In soybean cell cultures maximum PAL activity was found 7 to 8 hours after elicitation with a yeast carbohydrate preparation, maximum glyceollin synthesis followed 2 to 3 hours later, i.e. 10 to 12 hours after elicitation. At the same time, for a given cell line, different products may show highest levels of accumulation at different times, i.e., kievitone and phaseollin in Phaseolus vulgarus or phytuberrin and phytuberol in Nicotiana callus cultures.

Elicitors or elicitor preparations include both biotic and abiotic elicitors. Biotic elicitors intends those compounds of biological origin involved in plant-microbe interaction. The biotic elicitors are, in general, low molecular weight components of the microbial cell wall or extra cellular enzymes which induce plants to produce secondary metabolites as a cellular defense mechanism and includes homogenates, filtrates and extracts, including oligosaccharides, polysaccharides, glycoproteins and low molecular weight compounds, of fungal or bacterial origin. Pathogenic fungi, i.e., Phytophthora, Botrytis, Verticillium, etc., and non-pathogenic fungi, i.e. Aspergillus, Micromucor, Rhodotorula, etc. may find use. Bacterial elicitors include Rhizobium, Erwinia, Streptomyces, and the like. Table 1 gives examples of several elicitors as well as their means of preparation, the cell culture upon which they act and the products produced. Cultures show a significant increase in metabolite production after exposure to elicitors (Table 2).

Abiotic elicitors include stress agents such as UV light, alkalinity, osmotic pressure, heavy metal ions, and the like. Thus a wide variety of elicitors are known and can be utilized with the present method. Optimum employment of elicitor preparation for product accumulation requires the consideration of several criteria which may be optimized for each cell culture. These criteria include: elicitor specificity, elicitor concentration, duration of elicitor contact, cell line, time course of elicitation, growth stage of the culture,

growth regulation, and nutrient composition.

To determine the most efficient elicitor for any cell culture and particular metabolite, a variety of elicitor preparations may be screened. Certainly, where elicitors are known for a particular culture they may be utilized. The combination of more than one biotic elicitor to the elicitor preparation may find use as well as the combination of biotic and abiotic elicitors.

Protocols for the preparation of elicitors are known, such as is described in Eilert et al. J. Plant Physiol. (1985) 119: 65-76, whose disclosure is herein incorporated by reference. One such process for the preparation of fungal elicitors includes the excision of 1 cm^2 mycelium, inoculation of 100 ml of B5-medium without hormones, culture for seven days at room temperature, and homogenization of the entire culture followed with sterilization by autoclaving. Elicitor preparations can be further refined, if desired, by fractionation and purification.

The amount of elicitor preparation which is effective for the enhancement of secondary metabolite production varies widely and can be determined for each cell material. Maxium glyceollin synthesis in soybean cell cultures was obtained with a yeast carbohydrate preparation of 20 mg/g dry weight of cells.

The amount of time after elicitor contact for the accumulation of secondary metabolites will vary depending upon the elicitor preparation, the cell line being cultured, and the product being obtained. For the most part, only a relatively short exposure is necessary when considering products such as phytoalexins and other components of a plant's defense system. After elicitor contact the metabolites can be isolated by triggering the cells to release the stored metabolites into the medium, followed by an exchange of the culture medium, or by cell isolation with subsequent extraction of the metabolites. Thus, in general, elicitor contact will range from about 24 hours to about 60 hours preferably about 48 hours. This relatively shore duration of exposure necessary to induce maximum product accumulation is, indeed, a beneficial trait of elicitation.

Elicitors can be used alone or in combination with other means for increasing secondary metabolite production. These other methods include, but are not limited to, the use of a production medium, the variation of factors having a direct effect on secondary metabolite formation such as hormones or other chemical factors and variation of factors which affect the growth and viability of the cell cultures. It has been demonstrated (see Tables and Experimental Section) that the addition of cell viability stabilizers, and/or nutrients at the time of elicitation are particularly beneficial as they extend cell viability and maintain optimum secondary metabolite production following elicitor stress. Although these components improve the efficiency of metabolite production in general, they are especially important in situations where sudden product formation from elicitation is deleterious to plant cells.

Cell viability stabilizers include polyamines, osmolytes and the like. When polyamines are added along with elicitors, the transient increase in PAL activity following elicitation is stabilized and an increase in the amount of secondary metabolites formed following elicitation is seen (Tables 3 and 4). Polyamines are added for a final concentration of about 2 mM to about 10 mM, preferably about 5 mM. By polyamines is intended cadaverine, putrescine, spermidine, spermine, and the like. The use of tetramines are perhaps preferable as they are more effective than di- or triamines. Polyamines increase in plants following application of plant hormones and perhaps serve as secondary messengers similar to cAMP. Thus, the addition of plant hormones to the culture can have an influence on the growth and product formation of metabolites by increasing polyamines. In general, polyamines regulate physical and chemical properties in membranes and thereby alter permeability.

Organic osmolytes are cytoplasmic components which serve as osmoregulators and perhaps function primarily as protein stabilizers. Organic osmolytes include proline, ergosterol, glycerol, and the like. The addition of organic osmolytes following elicitation results in corresponding increases in secondary metabolite reduction (Tables 5 and 6). Osmolytes are demonstrated to be effective when added for a final concentration of about 0.5% w/v to about 2% w/v, preferably about 1% w/v.

For the purposes of the present invention, nutrients include macronutrients, micronutrients, vitamins, amino acids, hormones, growth regulators, carbohydrate sources, and the like. The variation of or addition of any of these components at the time of elicitation can result in improved yields of secondary metabolites. However, particularly beneficial results have been achieved by the addition of calcium (Table 7) and sucrose and potassium nitrate along with the elicitor. For calcium, a final concentration in the range of 1 mM to about 5 mM, preferably about 2 mM is preferred. For sucrose and potassium nitrate, final concentrations in the range of about 0.5% w/v to about 2% w/v, preferably 1% w/v, and about .05% w/v to about 0.3% w/v, preferably 0.1% w/v respectively, gives optimal results.

The addition of nutrients and/or stabilizers improved metabolite production when utilized independently at the time of elicitation. However, even greater yields may be accumulated by the addition of more than one component at the time of elicitation (see Tables 2 to 8). As discussed, optimum yields were found

when cell viability stabilizers, sucrose, potassium nitrate, and the elicitor was added. Therefore, although the addition of elicitors alone at the optimum time in the cell cycle enhances the production of secondary metabolites, significant increases can also be obtained by the utilization of cell viability stabilizers and/or nutrients at the time of elicitation.

The use of a two-stage culturing process using a second state of culturing in a liquid production medium (Zenk et al. In: Plant Tissue Culture and Its Biotechnological Application (1977). Barz et al. (eds.), pp. 27-43 and U.S. Patent No. 4,717,664) has been demonstrated to produce high levels of secondary metabolites in some cell cultures. The instant invention circumvents the need for the second stage of culturing in a production medium. The elicitation process provides for the substantial accumulation of metabolites without the imposition of special culture conditions or the transfer of cells to production media. However, if desired, elicitors may be used in combination with a production media process to shorten the culture period required for maximum product accumulation.

Metabolites, herein, are defined as elicitation products. These include a wide range of ubiquitous plant constituents, such as phenolic compounds, flavonoids, flavones, flavenones, phytoalexins, pterocarpans, alkaloids, and the like. These metabolites find use as proteinase inhibitors, plant virus inhibitors, enzymes, pigments, antitumor substances, plasma inhibitory compounds, antibiotics, spermicides, anti-inflammatory substances, etc. Metabolites or compounds which are attractive from the point of attempting to produce them by cell suspension culture include common and essential drug compounds derived from higher plants such as steroids, codeine, atropine, reserpine, digoxin, morphine, colchicine, etc. Besides these compounds which have been listed, there are of course numerous other compounds of pharmaceutical use which can be produced by the instant method. This includes the development of plant tissue and cell culture fermentation as a source for antineoplastic agents from cells of Maytenus, Brucea, Taxus, Baccharis and the like.

Aside from pharmaceuticals, the method finds use in the production of chemicals used in cosmetics, agrochemicals and natural aroma or flavor components from plant origin. The desired aroma compounds are mostly terpenoids which have one or more chiral centers and possess highly esteemed olfactory and taste properties. Chemical synthesis of these compounds is frequently not possible or at least not economical.

Cell cultures which are suitable for use include any which produce the metabolite of interest and respond to elicitors. Examples of the wide variety of cultures include, but are not limited, to those listed in Table 9.

For the industrialization of plant cell cultures to be realized the yield by which a given product is produced by fermentation per unit time must be economical. Important parameters in this regard are cell yield, product yield and growth. Secondary metabolites of plant cells are generally not excreted into the medium, but are retained and stored in the vacuole. The vacuole poses a natural limit as to the maximum yield of a given product to accumulate intracellularly. There are exceptions, however, as the excretion of high quantities of glutathione into the medium has been reported for tobacco cells. Likewise, products were detected in the respective media of periwinkle and poppy cells. While such occurrence could be due to excretion of the products into the media or to leakage due to cell breakdown, analysis of the ultrastructure of the cells following elicitation, revealed the cells to be perfectly intact. This finding points to excretion of the products into the culture medium rather than cell breakdown. Those cell cultures which do not naturally excrete metabolites into the culture meduim can be chemically induced to release their secondary metabolites. Chemical means for triggering the release of metabolites includes sorbitanoleate, taurocholate, dimethylsulfoxide, etc. Where necessary, one or a combination of these compounds can be added to the culture medium to trigger the release of secondary metabolites. Thus, one means for isolation of desired metabolites includes triggering the culture cells after elicitation to release the stored secondary metabolites into the medium. After release into the medium, the metabolites could be recovered without destruction of the culture cells. Thus, a preferred embodiment of the invention involves the cyclic process of cell growth and elicitation. Following elicitation, the cells, if necessary, are chemically triggered to release the elicitation products, the culture medium is collected, the metabolites extracted from the culture medium while permitting the plant cells to recycle followed by re-elicitation and extraction. The cycle is continued until a loss in metabolite formation is realized.

Other means for isolation of secondary metabolites includes isolation of the plant cells from the culture medium by gravity filtration, centrifuation and the like. Lysis of the plant cell membranes yields a concentrated slurry of the products from which the desired compounds can be extracted.

Another isolation embodiment includes the immobilization of cell cultures followed alternatively by treatment with elicitor and removal/exchange of medium for product recovery. As calcium alginate has demonstrated elicitor activity, entrapment of cells in calcium alginate followed by exchange of the medium

for product extraction may be employed.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1

Cell Lines and Culture Conditions

Suspension cultures of soybean cells (Glycine max) were derived from callus generated from soybean hypocotyls. The soybean cells were grown in the dark in Gamborgs B-5 medium with 0.5 ppm 2,4-D and 0.1 ppm of kinetin at 26° C and 125 rpm. Cells were subcultured every 7 days using a 20% (v/v) inoculum. The cells grew in clumps of 15-25 cells and sometimes larger aggregates. All experiments were incubated in 250 mL Erlemeyer flasks containing 100 mL medium. Every sampling was done in triplicate.

Example 2

Cell Dry Weight Estimation

Ten ml of actively growing culture was removed every day over the growth period. The sample was filtered under vacuum onto preweighed Whatman 1 filter paper. The paper was then oven dried for 24-36 hours at 70° C before being reweighed to determine the cell dry weight. Cell dry weight values were corrected to g/l of medium. The filtrate was retained and used for determination of peroxidase activity.

Example 3

Cell Dry Weight to Fresh Weight Correlation

About 70 samples from various stages of the growth cycle were measured for both their dry weight, which was determined gravimetrically prior to transferring samples to the oven. The correlation of dry weight to wet weight showed an average of 4.8% of fresh weight is made up of cell dry weight (60% of the estimations fell in the range of 4.8% - 4.9%).

Example 4

Peroxidase activity

Peroxidase activity was determined and correlated to cell dry weight. 4-Aminoantipyrine was used as the hydrogen donor in the peroxidase assay. The reaction rate was determined by measuring an increase in absorbance at 510 nm resulting from the decomposition of hydrogen peroxide and oxidation of the pyrine.

Procedure: 1.4 mL of phenol/antipyrine solution and 1.5 ml of 0.0017M hydrogen peroxide were mixed. The solution was then incubated for 3-4 minutes to achieve temperature equilibrium and establish the background reaction rate. 0.1 ml of extracellular filtrate (enzyme) was then added and the increase in absorbance at 510 nm was followed for 4-5 minutes. Activity was expressed as A_{510}/minute.

Example 5

Phenolic determination

0.5g (fresh wt %) cells (equivalent to 24 mg dry weight as determined by the correlation above) in 5 ml of 95% ethanol was sonically disrupted for 3-5 minutes. Afterwards the contents were centrifuged for 30 minutes at 12,000 rpm in a Beckman Model J21C centrifuge. The ethanol fraction (supernatant) was retained for the phenolic assay.

Assay: 1 ml of ethanol extract and 1 ml of 75% ethanol were mixed in 5 ml distilled water. then, 0.5 ml of 50% Folin-Ciocalteau reagent was added. After 5 min, 1 ml of 5% Na_2CO_3 was added, and the reaction mixture was allowed to stand for 60 min. Its absorbance was then measured at 725 nm. Controls contained only 95% ethanol. A standard curve was developed using various concentrations of gallic acid in 95% ethanol.

Example 6

Carbohydrate Estimation

One ml of cell free medium was assayed for carbohydrates by the anthrone method, Morris (1948) Science (1948) 107: 254. Carbohydrate depletion was estimated by measuring changes in absorbance of the anthrone color at 540 nm. from a standard curve using sucrose as the standard carbonydrate. Total carbohydrate in culture supernatants was expressed as grams of carbohydrate per liter of medium.

Example 7

Phenylalanine Ammonia Lyase Activity (PAL)

One gram of cells was suspended in 5 ml of pH 8.8 borate-HCl extraction buffer (25 mM borate-HCl + 2 mM sodium bisulfate). The suspension was gently ground with a mortar and pestle, the resulting mixture was sonically disrupted (1 minute; repeated 4-5 times at approximately 0° C). Between sonic disruptions the probe was cooled with ice for 30 seconds. After sonication, the homogenates were centrifuged at 10,000 rpm for 20 minutes, and the supernatant was then used as the enzyme extract.

Assay: 15 µM L-phenylalanine in 100 µM borate buffer (pH8.8) in a total volume of 2.8 ml was mixed with 0.2 ml of enzyme extract and incubated at 30° C. Controls contained 2.8 ml of 100 µM Borate HCl (pH 8.8) and 0.2 ml enzyme extract with no L-phenylalanine. During the first 30 minutes of incubation, there was a slow decrease in absorbance (290 nm) during stabilization of the reaction mixture. After stabilization, absorbance increase was followed at 290 nm for 60 minutes. Every assay was done in triplicate and corrected by subtraction of the corresponding control value. PAL activity was reported as milliunits (mu), where 1 mu equalled an absorbance change of 0.001 per minute per gram fresh weight of cells.

Example 8

Elicitors

Fusarim solani var. pisi.: One liter of late logarithmic phase cells grown in potato dextrose medium were centrifuged and, after determining their fresh weight, the cells were disrupted by blending and resuspended in 25 ml of 100mM potassium acetate (pH 5.5) in a 50 ml flask. This suspension was autoclaved (121° C: 15 min.). A 0.05% w/v equivalent of sterile elicitor was added to cell suspension cultures.

Microbial Cell Extracts as Elicitors in Phenolics Production

To obtain higher product concentration in plant cell cultures, separate growth media and product formation media can be used. We have circumvented this need and also reduced the time during which the optimum quantity of phenolics are produced by using microbial elicitors (Table 2). In this study cell extracts of fungus Fusarium solani var pisi, when added at the end of exponential growth (7 days) produced optimum phenolics in 8 days. L-Phenylalanine ammonia lyase activity transiently increased at 6 hours following elicitor addition.

Example 9

Growth and Phenolic Production

Growth parameters during exponential growth were determined using the equation:
$X = X_o e^{\mu_{max}(t-t_o)}$ where X is dry weight. Growth in batch culture was exponential from days 1 to 6 after one day lag phase. The lag phase was short due to the high inoculum level (20% v/v). The specific growth rate (μ_{max}) in the exponential phase was 0.21 day^{-1} corresponding to a doubling time of 3.3 days. Before the onset of stationary phase, the culture entered a declining growth rate period between 6-9 days. This declining growth rate was also reflected in a declining rate of carbohydrate utilization. The rate of carbohydrate utilization was rapid and approximately linear from the time of inoculation to day 7. After day 7, the rate of carbohydrate utilization shifted from 2.3 g/l-day to 1.0 g/l-day.

Ethanol soluble phenolics were produced in approximately equal amounts during the growth and the stationary phases. There was little phenolics production in the declining growth phase and after day 12. After an additional 3 days, the phenolics concentration had increased only 15% from the level observed at day 12.

Example 10

Extracellular Peroxidase and L-Phenylalanine Ammonia Lyase (PAL Activity during Growth)

The rate of extracellular peroxidase activity as an indicator of cell growth was also examined. It was found that peroxidase activity was correlated with growth. The leveling off of peroxidase activity coincided with the changes in growth kinetics, rate of carbohydrate utilization, and phenolic accumulation. A most interesting observation was that optimum phenolic formation as a result of elicitation was triggered when elicitors were added at the end of 7 days of cell growth. Adding elicitor earlier than 7 days was not feasible because of low cell mass, or beyond 7 days because the enzymes needed to respond to elicitors and turn

on phenolic production had reduced activities, as exemplified by the peroxidase activity. Also, with the onset of senescence, there was reduced cell-division as cells entered stationary phase.

L-Phenylalanine ammonia-lyase (PAL) activity was followed over the growth period. PAL activity increased at the onset of stationary phase coinciding with the onset of soluble phenolics production. PAL is a key enzyme of the phenylpropanoid pathway and many of these compounds are formed when cell division ceases.

Example 11

Role of Calcium, Polyamines and Osmolytes in Cellular Production of Phenolics

Calcium: Calcium increased activity of PAL and concomitantly increased total phenolics when added along with the elicitor (Table 3-7). The transient increase in PAL was similar to what was observed by elicitor alone though overall activity was higher. Changes in calcium concentration affected the elicitor mediated phytoalexin accumulation, and by removal of all calcium with chelating agents, elicitor mediated enzyme induction and phytoalexin production were eliminated.

Polyamines The polyamines spermidine and spermine when added along with calcium and elicitors stabilized the transient increase in PAL activity following elicitation. They also increased the amount of phenolics formed compared to using only calcium and elicitors (Tables 3 and 4). It appears that the quality of phenolics formed may be affected by polyamines. It has been observed that spermidine not only extended the life span of mature non-dividing cells but also increased yields of phenolics and gave a wider assortment of phenolics, Muhitch et al (1985) Plant Physiol (1985) 78: 25.

Organic Osmolytes: Organic osmolytes are cytoplasmic components serving as osmoregulators and perhaps working primarily as protein stabilizers. The organic osmolyte proline used in this study did not stabilize the transient increase in PAL activity following elicitor and calcium addition. However, proline gave an increase in phenolics produced comparable to adding elicitor and calcium alone. (Tables 5 and 6). The organic osmolyte glycerol stabilized the transient increase in PAL activity following elicitor addition. The addition of glycerol also resulted in a corresponding increase in phenolics (Table 8).

Addition of Sucrose and a Nitrogen Source During Elicitation for Phenolics Production: Phenolics production in Glycine max cell suspension was highest when elicitor was added with a cell viability stabilizer, sucrose and potassium nitrate (Table 7).

The instant method offers several advantages for the production of secondary metabolites through cell culture. Current methods for the production of secondary metabolites requires culturing the cells first in a growth media followed by culturing in a product formation media. The present invention circumvents this need for a growth media and production formation media. The use of only one culture media reduces labor and cost of the production of metabolites, as well as reduces the time during which the optimum quantity of metabolites are produced.

The use of elicitors to enhance secondary metabolite formation offers a powerful tool for the production of specific compounds through tissue culture. Improved yields of metabolites are obtained by elicitation alone. Further maximization can be realized by the addition of cell viability stabilizers and/or nutrients at the time of elicitation.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent appliction was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claim.

Table 1

ELICITOR-INDUCED PRODUCT ACCUMULATION			
Elicitor	Preparation	Cell Culture	Products
Pythium aphanidermatum Eurotium rubrum Micromucor isabellina Chrysosporium palmorum	Filtrates and extracts	Catharanthus roseus	Tryptamine Ajmalicine Catharanthine
Pythium aphanidermatum	Homogenate	Catharanthus roseus	Strictosidine Ajmalicine Tabersonine Lochmericine Catharanthine
Botrytis spec.	Homogenate	Papaver somniferum	Sanguinarine
Dendryphion spec.	Extract	Papaver somniferum Papaver bacteatum	Sanguinarine Sanguinarine
Yeast	Carbohydrate preparation	Glycine max Thalictrum rugosum	Glyceollin Berberine
Aspergillus niger	Homogenate	Cinchona ledgeriana Rubia tinctoria Morinda citrofolia	Anthraquinones
Nigeran		Solanum melongena Vigna angularis	Polyacetylenes Isoflavones

Table 2

Elicitor Responses as Measured by Soluble Phenolics (mg/l) after Addition of Microbial Elicitors (Fusarim solani var. pisi)			
	Time (h)		
	0	24	48
Elicitor	61.7	123.1	137.3[a]
Control (no addition)	63.4	62.1	67.4

[a]Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

Table 3

Elicitor Responses as Measured by Soluble Phenolics (mg/l) after Addition of Microbial Elicitors: (Fusarium solani var. pisi)			
Effect of Calcium (2 mM) and Spermidine (5 mM)			
	Time (h)		
	0	24	48
Elicitor + Ca^{++} + Spermidine	63.7	127.8	158.6[a]
Elicitor + Ca^{++}	61.8	118.7	143.6
Elicitor + Spermidine	63.9	116.3	141.3
Elicitor	64.6	123.6	133.7
Control + Ca^{++} + Spermidine	59.2	63.4	62.7
Control + Ca^{++}	67.3	64.6	65.3
Control + Spermidine	58.6	61.4	63.5
Control	63.7	61.6	62.3

[a]Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

Table 4

Elicitor Responses as Measured by Soluble Phenolics (mg/l) after Addition of Microbial Elicitors: (Fusarium solani var. pisi)			
Effect of Calcium (2 mM) and Spermine (5 mM)			
	Time (h)		
	0	24	48
Elicitor + Ca^{++} + Spermine	72.3	123.7	158.6[a]
Elicitor + Ca^{++}	69.6	124.2	143.3
Elicitor + Spermine	63.7	118.7	140.8
Elicitor	68.7	120.3	131.4
Control + Ca^{++} + Spermine	63.6	70.4	69.8
Control + Ca^{++}	64.8	67.3	68.1
Control + Spermine	61.2	62.8	70.3
Control	69.1	67.4	62.8

[a]Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

Table 5

Elicitor Responses as Measured by Soluble Phenolics (mg/l) after Addition of Microbial Elicitors: (Fusarium solani var. pisi)			
Effect of Calcium (2 mM) and Proline (5 mM)			
	Time (h)		
	0	24	48
Elicitor + Ca^{++} + Proline	71.4	117.3	158.1[a]
Elicitor + Ca^{++}	63.8	119.7	141.6
Elicitor + Proline	61.3	118.8	147.3
Elicitor	63.7	121.7	138.8
Control + Ca^{++} + Proline	69.8	63.8	64.3
Control + Ca^{++}	63.9	65.6	67.8
Control + Proline	69.8	69.3	68.4
Control	70.6	71.4	72.3

[a]Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

Table 6

Elicitor Responses as Measured by Soluble Phenolics (mg/l) after Addition of Microbial Elicitors: (Fusarium solani var. pisi)			
Effect of Calcium (2 mM) and Glycerol (1% w/v)			
	Time (h)		
	0	24	48
Elicitor + Ca^{++} + Glycerol	53.4	123.7	160.4[a]
Elicitor + Ca^{++}	54.3	119.3	143.3
Elicitor + Glycerol	59.8	129.6	153.3
Elicitor	57.4	123.8	141.7
Control + Ca^{++} + Glycerol	61.8	63.8	67.4
Control + Ca^{++}	59.6	61.7	62.2
Control + Glycerol	57.6	59.3	58.4
Control	63.4	61.8	67.6

[a]Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

Table 7

Elicitor Responses as Measured by Soluble Phenolics (mg/l) after Addition of Microbial Elicitors: (Fusarium solani var. pisi)			
Effect of Calcium (Ca^{++})			
	Time (h)		
	0	24	48
Elicitor	63.4	117.4	139.8
Ca^{++}	61.3	63.4	64.6
Elicitor + Ca^{++}	59.7	123.7	149.8[a]
Control (no addition)	58.7	61.3	62.7

[a]Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

Table 8

Elicitor Responses as Measured by Soluble Phenolics (mg/l) after Addition of Microbial Elicitors: (Fusarium solani var. pisi)		
Effect of Calcium, Polyamines/Osmolytes and Sucrose + KNO_3		
	Time (h)	
	0	24
Control	57.8	64.7
Sucrose + KNO_3	68.4	63.4
Elicitor + (Sucrose + KNO_3)	63.4	154.7
Elicitor + Ca^{++} + (Sucrose + KNO_3)	59.3	159.8
Elicitor + Ca^{++} + (Sucrose + KNO_3) + Spermine	63.3	160.4
Elicitor + Ca^{++} + (Sucrose + KNO_3) + Spermidine	59.5	171.3[a]
Elicitor + Ca^{++} + (Sucrose + KNO_3) + Proline	65.4	159.7
Elicitor + Ca^{++} + (Sucrose + KNO_3) + Glycerol	59.6	174.6[a]

[a]Significant at 95% confidence level from all other treatments using Fisher's L.S.D. All values are average of three readings.

Table 9

Metabolites and the Cell Cultures From Which They Are Produced	
Product	Plant Species
Ginsengoside	Panax ginseng
Anthraquinones	Morinda citrifolia
Rosmarinic acid	Coleus blumei
Shikonin	Lithospermum erythrorhizon
Anthraquinones	Cassia tora
Diosgenin	Dioscorea deltoides
Biscoclaurine	Stephania cepharantha
Caffein	Coffea arabica
Ajmalicine	Catharanthus roseus
Paniculide B	Andrographis paniculate
Serpentine	Catharanthus roseus
Protopine	Macleaya microcarpa
Visnagin	Ammi visnaga
Glutathione	Nicotiana tabacum
Ubiquinone-10	Nicotiana tabacum
Alkaloids	Catharanthus roseus
Nicotine	Nicotiana rustica
Alkaloids	Solanum laciniatum
Catechols	Ilex pupescens
Catechols	Artemisia capillaris
Catechols	Salvia miltiorrhiza
Artemisian	Artemisia annua
Betacyanin	Beta vulgaris
Tannin	Krameria triandra
Tannin	Arctostaphylos upa-uris
Cinnamic acid derivatives	Artemesia tridentata
Berberine	Coptis japonica
Vanillin	Vanilla planifolia

Claims

1. A method for enhancing secondary metabolite production from a plant cell culture, said method comprising:
growing a plant cell culture in a production medium to about the end of exponential growth phase;
adding to said medium an elicitor preparation in an amount to enhance secondary metabolite production; and
isolating at least one secondary metabolite.

2. A method, according to Claim 1, wherein said end of exponential growth phase is determined by monitoring extracellular products.

3. A method, according to Claim 2, wherein said monitoring comprises assaying for extracellular peroxidase activity.

4. A method for enhancing secondary metabolite production from a plant cell culture, said method comprising:
growing a plant cell culture in a production medium to at least about the end of exponential growth phase;
adding to said medium an elicitor preparation and at least one of cell viability stabilizers and nutrients in amounts to enhance and stabilize secondary metabolite production; and,
isolating at least one secondary metabolite.

5. A method, according to Claim 4, wherein said end of exponential growth phase is determined by

assaying for extracellular peroxidase activity.

6. A method, according to claim 4 or claim 5, wherein said viability stabilizers comprise polyamines and osmolytes.

7. A method, according to Claim 6, wherein said polyamines are selected from spermidine, spermine, cadaverine, and putrescine.

8. A method, according to Claim 7, wherein said polyamine is spermidine.

9. A method, according to Claim 7, wherein said polyamine is spermine.

10. A method, according to Claim 6, wherein said osmolytes are selected from proline, ergosterol and glycerol.

11. A method, according to Claim 10, wherein said osmolyte is proline.

12. A method, according to Claim 10, wherein said osmolyte is glycerol.

13. A method, according to claim 4 or claim 5, wherein said nutrients are selected from sucrose, calcium, and potassium nitrate.

14. A method, according to claim 4 or claim 5, wherein said adding step comprises the addition of an elicitor preparation, calcium, sucrose, postassium nitrate and at least one of spermine, spermidine, proline and glycerol.

15. A method, according to any one of the preceding claims wherein said secondary metabolite is flavonoids, flavones, flavonones, phytoalexins, pterocarpins, and alkaloids.

16. A method, according to any one of the preceding claims, wherein said plant cell cultures are produced from plants of Boraginaceae, Pyrolaceae, Juglandaceae, Plumbaginaceae, Lythraceae, Ranunculaceae and Solanaceae.

17. A method, according to Claim 16, wherein said secondary metabolite is artemesian and said plant cell culture is Artemesia annua.

18. A method, according to Claim 16, wherein said secondary metabolite is betacyanin and said plant cell culture is Beta vulgaris.

19. A method, according to Claim 16, wherein said secondary metabolite is vanillin and said plant cell culture is Vanilla planifolia.

20. A method, according to Claim 16, wherein said secondary metabolite is rosmarinic acid and said plant cell culture is Coleus blumei.

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(54) PROCESS FOR PRODUCING TAXOL BY CELL CULTURE OF TAXUS SPECIES

VERFAHREN ZUR HERSTELLUNG VON TAXOL MITTELS TAXUSSPEZIES-ZELLKULTUR

PROCEDE POUR PRODUIRE DU TAXOL PAR CULTURE CELLULAIRE DE L'ESPECE DE TAXUS

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Description

The present invention relates to cell culture of plants belonging to the genus Taxus. More specifically, the present invention relates to a process for producing taxol by cell culture of plants belonging to the genus Taxus.

Taxol is an alkaloid of diterpene, which exhibits a carcinostatic activity and inhibits cytokinesis.

Taxol is a compound promising as an antitumor agent. In particular, for ovarian cancer, taxol has been subjected to the Phase III clinical trial sponsored by the National Cancer Institute (NCI) of National Institute of Health in the U.S.A. In the known process, taxol is produced by an extraction from the bark of the wild Pacific Yew tree, Taxus brevifolia NUTT. However, since the Pacific Yew plants grow very slowly, the regrowth of the bark after removal thereof is not expected, and further, the content of taxol therein, and the production efficiency thereof, are very low. For example, as much as 10 tons of the bark are required for producing as little as 1 kg of taxol. Accordingly, it is estimated that 2000 to 4000 Pacific Yew trees, which are 50 to 250 years old, must be cut down to yield 1 kg of taxol. Consequently, many companies and research institutes are attempting to develop alternative processes for producing taxol.

As a substitute for extracting the alkaloid from rare plant sources, there can be mentioned a process for extracting the desired alkaloid from cultured cells from the objective plant tissues grown in an artificial growth medium, but no example of a successful production and/or isolation of taxol from cultured cells of the plant belonging to the genus Taxus has been described in any literature. For example, M. A. Zenkteler et. al. (Acta. Soc. Bot. Pol., 39 (1): pp. 161-173, (1970)) disclose the formation of calli from female gametophytes of Taxus baccata LINN., but there is no description of whether or not the disclosed callus formation protocols can be applied to other species of the genus Taxus, and no description of whether or not the calli produce any alkaloid. Also there is no description teaching such matters. Nevertheless, research into the development of processes for producing taxol by cell culture has not been abandoned, since cell culture is still thought to be feasible as a process for producing same.

Abstract no. 2252 on p. 566 of vol. 30 of the Proceedings of the Eightieth Annual Meeting of the American Association of Cancer Research 24-27 May 1989 and US-A-5,019,504 both disclose a method for taxol production in which

tissue explants of Taxus brevifolia were placed on solid media;
the resulting cell lines were grown in suspension culture; and
taxol was obtained from the culture extract.

However, the described methods do not lead to an efficient production of taxol by the cultured cells.

Therefore, the object of the present invention is, to provide a process for efficiently producing taxol by cell culture of Taxus plant.

To attain the object described above, the present inventors have made intensive research into callus initiation from tissues of plants belonging to the genus Taxus and into proliferation of the callus cells. As a result, it has been found that cultured cells having a high taxol content can be efficiently obtained by culturing specific tissues originating from specific species belonging to the genus Taxus, whereby the present invention was achieved.

Namely, according to the present invention, there is provided a process for producing taxol by culturing cells originating from tissues of plants belonging to the genus Taxus, which comprises:

a) a step of preparing a living tissue;
b) a step of culturing the tissue obtained in step a) in a nutrient medium containing a gibberellin suitable for inducing a callus, to thereby induce a callus;
c) a step of culturing the callus cells obtained in step b) in a nutrient medium suitable for proliferating suspension culture cells; and
d) a step of recovering taxol from the cultured product obtained in step c).

Also provided are a process for inducing and proliferating the callus using some of the above-described steps, and the callus or suspension culture cells containing at least taxol as an alkaloid.

The phrase "plants belonging to the genus Taxus", as used in the present invention, means plants belonging to the genus Taxus of the family Taxaceae, and suitable for the object of the present invention (i.e. production of taxol). Typical examples thereof include, but are not restricted to, Pacific Yew (Taxus brevifolia NUTT.) and Japanese Yew (Taxus cuspidata SIE3. et ZUCC.).

According to the present invention, living tissues are prepared from specific tissues of these plants in a first stage. Consequently, as a piece of plant material put into culture, shoots, leaves, roots, flowers, fruits and seeds can be used as it is, or the plant materials may include those prepared in a form suitable for the culture, for example, in the case of the seeds, clean germinating tissues isolated from surface sterilized seeds. Of these leaves, shoots and female gametophytes, which are nutrient-storage tissues of a Gymnosperms seed, are preferable for the present invention. The endosperms or female gametophytes have been hitherto used for studies of tripoid or haploid plant development, and

for studies of the bio-synthesis of seed storage nutrients. Surprisingly, the female gametophytes are found particularly suitable for initiating a callus and producing suspension culture cells containing considerable amounts of taxol. The above-described preparation is part of the concept of including treatments in which the plant materials for culture are isolated as they are and alive, and are surface sterilized. Typical examples of such preparations involve, but are not limited to, steps in which, first the materials collected from plants of the genus Taxus are sterilized with 70% ethanol and an aqueous solution of sodium hypochlorite, the raw materials are then aseptically divided into pieces, and thereafter, are transferred onto media solidified by agar or gellan gum suitable for inducing a callus as described hereinbelow.

The living tissues obtained in the above-described stage are then cultured on a nutrient media containing a gibberellin suitable for callus induction, and the calli thus derived are transferred to a nutrient medium suitable for the proliferation of callus cells. The primary components to be used in the nutrient medium include water; mineral nutrients, e.g. nitrogen (ammonium salts and nitrates), phosphorus, potassium, calcium, magnesium, sulfur, etc.; sugars, e.g., sucrose, glucose, fructose, maltose, etc.; organic substances such as vitamins and amino acids; naturally originating substances such as coconut milk; and optionally, gelling agents, e.g., agar, gellan gum, alginic acid, and agarose. The basic formulations of mineral nutrients known as basic media include Schenk & Hildebrandt medium (hereinafter referred to as "SH medium"), Murashige & Skoog medium (hereinafter referred to as "MS medium"), Gamborg's B5 medium, White's medium, Nitsch & Nitsch medium, Nagata & Takebe medium and woody plant medium. As regards media for proliferating callus cells a gibberellin (hereinafter referred to as "GA", such as GA1 and GA3) is added to these basic media and further optionally auxins such as 1-naphthaleneacetic acid (hereinafter referred to as "NAA"), indole-3-acetic acid (hereinafter referred to as "IAA"), indole-3-butyric acid (hereinafter referred to as "IBA"), and 2,4-dichlorophenoxyacetic acid (hereinafter referred to as "2,4-D") or cytokinins such as benzylaminopurine (hereinafter referred to as "BA"), zeatin, and 6-furfurylaminopurine (kinetin); are added as plant growth regulators. As the nutrient media suitable for inducing the callus according to the present invention, media in which a gibberellin and optionally auxins and/or cytokinins are added to the above-described basic media are preferable. Typically, a medium using the SH medium as the basic medium to which BA as the cytokinin and GA_3 as the gibberellin are added, is preferable. On the other hand, as the nutrient media suitable for proliferating suspension culture cells, the above-described basic media with cytokinins and auxins added thereto are used, with the media using the SH medium as the basic medium and having kinetin as the cytokinin and NAA or 2,4-D as the auxin added thereto being particularly preferable. The pH levels of such media are adjusted to 5 to 7, preferably 5.5 to 6.0, with an appropriate acid or alkali.

Each of the above-described cultures using these media can be carried out at a temperature in the range of 15-25°C with or without light-irradiation. In particular, the cultures for the proliferation of the callus cells are preferably carried out in a liquid medium, in which the medium containers are placed on a rotary shaker.

The isolation of taxol thus obtained from the cultured products can be carried out by following the method of extracting various alkaloids from cultured tissue followed by purification, which is known per se. The term "cultured products" used herein is intended to include calli, cultured cells, clumps of cultured cells, cultured tissue, cultured organs and a medium used for the culture. Although not restricted thereto, the taxol can be isolated from such cultivated products by separating calli or cultured cells from media, drying and pulverizing same, extracting taxol from the resulting powder with an appropriate organic solvent, optionally washing the organic phase with water, drying the organic solvent over anhydrous sodium sulfate, magnesium sulfate or calcium chloride, and then evaporating the solvent. If necessary, the taxol thus isolated can be purified by various chromatographic purifications or recrystallization, etc. Examples of an organic solvent which can be used in the extraction include chlorinated hydrocarbons such as methylene chloride and dichloroethane; and alcohols such as methyl, ethyl and isopropyl alcohol.

The substance isolated by the process described above has been confirmed to be taxol, by comparing the NMR, IR, MASS, and UV spectrums thereof with those of a standard sample of taxol received from the above-described NCI.

According to the present invention, the calli or cultured cells having a high taxol content can be effectively obtained by the cell culture of the plants belonging to the genus Taxus (for example, about 10 g of cells in dry matter was obtained from a liter of the suspension culture, and the cells thus obtained had a taxol content of 0.05%.) The taxol content of the dried callus was approximately 10 times greater than that of the dried bark of Pacific Yew trees.

The calli or suspension cells containing taxol may be used as an antitumor agent as are, by isolating and pulverizing same.

EXAMPLES

The present invention will now be described in greater detail by referring to working examples 1, 3 and 4. Examples 2 and 5 are not within the scope of the claims.

Example 1

A seed (5 g) of Taxus brevifolia NUTT from which aril had been removed was immersed in an aqueous solution

of 70% W/W ethanol (50 mℓ) for 10 to 30 seconds, and then in an aqueous sodium hypochlorite solution having an effective chlorine concentration of 1% (50 mℓ) for 20 minutes, and there after, was rinsed three times in sterile water (100 mℓ) to eliminate surface contaminants. After the sterilization, the hard external seed coat and the thin internal seed coat were removed with tweezers, and resulting embryos and female gametophytes were then excised and used as an explant.

To prepare medium for culture of an explant obtained as described above, SH medium (supplemented with 0.25% by weight of gellan gum) containing 1, 5 or 10 mg/ℓ of BA as a growth regulator, and having a pH value adjusted to 5.8 with potassium hydroxide or hydrochloric acid, was sterilized by using an autoclave at temperature of 120°C for 15 minutes, and thereafter, GA_3 (separately sterilized by passing through a membrane filter) was added to concentrations of 0, 1, 10 or 100 mg/ℓ. The medium was dispensed into test tubes and solidified, in which the explants were transferred and cultured at a temperature of 20° or 25°C under a light (16 hours per day), to thus induce in vitro germination of an embryo or callus initiation. It was found that the callus was induced from female gametophytes after approximately 2 months of culture. The results of the callus induction are shown in Table 1.

Table 1: Results of Culture

(Regulator concentration: mg/ℓ)

BA \ GA_3	20°C 0	20°C 1	20°C 10	20°C 100	25°C 0	25°C 1	25°C 10	25°C 100
1	△	○	△	△	△	○	△	△
5	△	○	x	△	△	○	x	x
10	△	○	x	x	△	○	x	x

○: Initiation and proliferation of callus from female gametophytes

△: No initiation of callus

x: Death

Example 2

A leaf (5 g) and a shoot (5 g) of Taxus cuspidata SIEB. et ZUCC. were immersed in an aqueous solution of 70% W/W ethanol (50 mℓ) for 15 seconds, and then in an aqueous sodium hypochlorite solution having 1% of an effective chlorine concentration for 10 minutes, and there after, were rinsed three times with sterile water (100 mℓ) to eliminate surface contaminants. After the sterilization, the leaf and the shoot were cut to lengths of approximately 5 mm.

The explants thus prepared were transferred to MS medium (supplemented with 0.25% by weight of gellan gum) containing NAA and kinetin as plant growth regulators in the concentrations as shown in Tables 2 and 3, and having a pH value adjusted to 5.8 with potassium hydroxide or hydrochloric acid, and were then cultured at a temperature of 20° or 25°C in the dark or under a light (16 hours per day) to induce a callus. As shown in Table 4, the callus was induced at particular combinations of two plant growth regulator and sucrose concentrations.

Table 2: Combinations of Plant Growth Regulator Concentrations in MS Medium Used for Callus Initiation

(Medium containing 30g/ℓ of sucrose)

NAA \ Kinetin	0.5	1.5	5.0
1.0	(1)	(2)	(3)
4.0	(4)	(5)	(6)
10.0	(7)	(8)	(9)

(Regulator concentration Unit: mg/ ℓ)

(Number in parentheses indicates medium number used for callus initiation experiments.)

Table 3: Combinations of Plant Growth Regulator Concentrations in MS Medium used for Callus Initiation

(Medium containing 10g/ℓ of sucrose)

NAA \ Kinetin	0.0	0.1	0.5
1.0	(10)	(11)	(12)
4.0	(13)	(14)	(15)
10.0	(16)	(17)	(18)

(Regulator concentration Unit: mg/ ℓ)

(Number in parentheses indicates medium number used for callus initiation experiments.)

Table 4:

Results of Callus Induction after 2 months of culture				
Medium No.	Leaf		Stem	
	Light	Dark	Light	Dark
(1)	x	x	o	Δ
(2)	x	x	x	x
(3)	x	x	x	x
(4)	x	x	Δ	x
(5)	x	x	o	x

Table 4: (continued)

Results of Callus Induction after 2 months of culture				
Medium No.	Leaf		Stem	
	Light	Dark	Light	Dark
(6)	x	x	Δ	x
(7)	x	x	x	x
(8)	Δ	x	x	x
(9)	x	x	x	x
(10)	x	x	Δ	x
(11)	x	x	x	x
(12)	x	x	x	x
(13)	x	x	x	x
(14)	Δ	x	o	x
(15)	x	x	o	x
(16)	x	x	x	x
(17)	Δ	x	x	x
(18)	x	x	x	x
o: Initiation and proliferation of callus Δ: No initiation of callus x: Death				

Example 3

The callus induced from female gametophytes in Example 1 was transferred to SH medium (liquid medium) containing 5 mg/ℓ of NAA and 0, 0.1 or 5 mg/ℓ of kinetin as plant growth regulators and having a pH value adjusted to 5.8. Erlenmeyer 100 mℓ flasks each containing 40 mℓ of the medium were used for the culture. The flasks were placed on a rotary shaker and shaken at a rate of 100 rpm, and at a temperature of 20°C, in the dark.

After 4 weeks of culture, it was observed that, in the medium containing 5 mg/ℓ of NAA alone as a growth regulator, the cell mass increased by about 5 times.

Cell growth in the medium containing 5 mg/ℓ of NAA was examined at a temperature of 20° or 25°C in the dark or under a light (16 hours per day). Although cell mass increased under all test conditions, the largest growth rate was observed at a temperature of 20°C in the dark.

When 2,4-D was used as the auxin in place of NAA, similar results were obtained.

Example 4

The cells grown in Example 3 were collected and air-dried. The extraction and purification of taxol from the cells were carried out with reference to a process for extraction of taxol from the bark of a Taxus plant (M. Keith, J. Natural Products, Vol. 53 (5), pp. 1249-1255, 1990). The air-dried cells were pulverized, and extracted with methylene chloride-methanol (1 : 1). The extraction solution was separated from the cell debris and evaporated, and the residue was then suspended in methylene chloride. This organic suspension was washed with water, and the solvent was then evaporated. The resulting residue was resuspended in methanol, and taxol was isolated and purified from the methanol suspension by High Performance Liquid Chromalography (HPLC) equipped with a reverse phase column (M. Keith, J. Liquid Chromatography, Vol. 12, pp. 2117-2132, 1989).

The identification of taxol from the cell culture was carried out by comparing a retention time of peaks obtained for the above-mentioned methanol suspension by HPLC with that of a standard sample of taxol from NCI, and as a result, a peak with an identical retention time with the standard sample was observed. The chemical structure of the substance purified from the methanol suspension by HPLC was determined by a mass spectrum analysis and NMR, and it was confirmed that the substance was taxol. The taxol content was 0.05% of dry matter of suspension cells. This content was larger than that of dried bark by approximately 10 times. (M. Keith, J. Natural Products, Vol. 53 (5), pp. 1249-1255, 1990).

Example 5

The callus induced from the shoot in Example 2 was transferred to SH medium (liquid medium) containing 5 mg/ℓ of NAA and 0, 0.1, or 5 mg/ℓ of kinetin as plant growth regulators and having a pH value adjusted to 5.8. Erlenmeyer 100 mℓ flasks each containing 40 mℓ of the medium were used for the culture. The flasks were placed on a rotary shaker and shaken at a rate of 100 rpm, and at a temperature of 20°C, in the dark.

After 3 weeks of culture, the callus cells were proliferated only in the medium containing 5 mg/ℓ of NAA alone, at a temperature of 20°C in the dark.

INDUSTRIAL APPLICABILITY

According to the present invention, callus cells of the Taxus plants containing at least taxol as alkaloids can be effectively induced and proliferated with artificial growth media. Furthermore, taxol can be isolated from these cultured cells. Accordingly, the present invention is available for use in the field of manufacturing medicinal preparations possessing a carcinostatic activity.

Claims

1. A process for producing taxol by culturing cells originating from tissues of plants belonging to the genus Taxus, which comprises:

   a) a step of preparing a living tissue;
   b) a step of culturing the tissue obtained in stage a) in a nutrient medium containing gibberellin and suitable for inducing a callus, to thereby induce a callus;
   c) a step of culturing the callus cells obtained in step b) in a nutrient medium suitable for proliferating suspension culture cells ; and
   d) a step of recovering taxol from the cultured products obtained in step c).

2. The process according to claim 1, wherein the plant species belonging to genus Taxus is Taxus brevifolia NUTT.

3. The process according to claim 1, wherein the plant belonging to genus Taxus is Taxus cuspidata SIEB. et ZUCC.

4. The process according to any one of claims 1 to 3, wherein the tissue is selected from the group consisting of leaves, shoots and female gametophytes.

5. The process according to any one of claims 1 to 4, wherein the cultivated product is suspension culture cells.

6. The process according to any one of claims 1 to 5, wherein the nutrient medium suitable for inducing the callus is SH medium to which benzyladenine and gibberellin have been added as plant growth regulators.

7. The process according to any one of claims 1 to 6, wherein the nutrient medium suitable for proliferating the suspension culture cells is SH medium to which the combination of kinetin with naphthaleneacetic acid or of kinetin with 2,4-dichlorophenoxyacetic acid has been added as plant growth regulators.

8. A process for inducing a callus and proliferating suspension culture cells comprising steps of a) to c) of claim 1, a female gametophyte of Taxus brevifolia NUTT. or Taxus cuspidata SIEB. et ZUCC. being used as the living tissue.

9. A callus or suspension culture cells obtained by the process of claim 8, which contains at least taxol as alkaloids.

Patentansprüche

1. Verfahren zur Herstellung von Taxol durch Züchtung von Zellen, die aus Gewebe von Pflanzen stammen, die zur Gattung Taxus gehören, umfassend die Schritte:

   a) Herstellung eines lebenden Gewebes;
   b) Züchtung des Gewebes aus a) in einem Nährmedium, das Gibberellin enthält und für die Induktion von

Callus geeignet ist, um Callus zu induzieren;
c) Züchtung der Calluszellen von b) in einem Nährmedium, das für die Vermehrung einer Suspensionszellkultur geeignet ist; und
d) Gewinnung von Taxol aus den gezüchteten Produkten von c).

2. Verfahren nach Anspruch 1, wobei die Pflanzenart, die zur Gattung Taxus gehört, Taxus brevifolia NUTT. ist.

3. Verfahren nach Anspruch 1, wobei die Pflanze, die zur Gattung Taxus gehört, Taxus cuspidata SIEB, et ZUCC. ist.

4. Verfahren nach einem der Ansprüche 1 bis 3, wobei das Gewebe ausgewählt ist aus Blättern, Sprossen und weiblichen Gametophyten.

5. Verfahren nach einem der Ansprüche 1 bis 4, wobei das gezüchtete Produkt eine Suspensionszellkultur ist.

6. Verfahren nach einem der Ansprüche 1 bis 5, wobei das Nährmedium, das für die Callusinduktion geeignet ist, SH-Medium ist, zu welchem Benzyladenin und Gibberellin als Pflanzen-Wachstumsregulatoren zugegeben wurden.

7. Verfahren nach einem der Ansprüche 1 bis 6, wobei das Nährmedium, das für die Vermehrung der Suspensionszellkultur geeignet ist, SH-Medium ist, zu welchem eine Kombination von Kinetin mit Naphthalinessigsäure oder Kinetin mit 2,4-Dichlorphenoxyessigsäure als Pflanzen-Wachstumsregulatoren zugegeben wurde.

8. Verfahren zur Induktion von Callus und zur Vermehrung einer Suspensionszellkultur, umfassend die Schritte a) bis c) von Anspruch 1, eine weibliche Gametophyte von Taxus brevifolia NUTT, oder Taxus cuspidata SIEB. et ZUCC., die als das lebende Gewebe verwendet werden.

9. Callus oder Suspensionszellkultur, erhalten durch das Verfahren nach Anspruch 8, welche zumindest Taxol als Alkaloid enthalten.

Revendications

1. Procédé pour produire du taxol par culture de cellules provenant de tissus de végétaux appartenant au genre Taxus, qui comprend :

a) une étape de préparation d'un tissu vivant ;
b) une étape de culture du tissu obtenu dans l'étape a) dans un milieu nutritif contenant une gibbérelline et convenant pour induire un cal, pour induire ainsi un cal ;
c) une étape de culture des cellules de cal obtenues dans l'étape b) dans un milieu nutritif convenant pour faire proliférer des cellules en culture en suspension ; et
d) une étape de récupération du taxol à partir des produits cultivés obtenus dans l'étape c).

2. Procédé selon la revendication 1, dans lequel l'espèce végétale appartenant au genre Taxus est Taxus brevifolia NUTT.

3. Procédé selon la revendication 1, dans lequel le végétal appartenant au genre Taxus est Taxus cuspidata SIEB et ZUCC.

4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel le tissu est choisi dans le groupe comprenant les feuilles, les pousses et les gamétophytes femelles.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel le produit cultivé est constitué par des cellules en culture en suspension.

6. Procédé selon l'une quelconque des revendications 1 à 5, dans lequel le milieu nutritif convenant pour induire le cal est du milieu SH auquel de la benzyladénine et une gibbérelline ont été ajoutées comme régulateurs de croissance des végétaux.

7. Procédé selon l'une quelconque des revendications 1 à 6, dans lequel le milieu nutritif convenant pour faire proliférer les cellules en culture en suspension est du milieu SH auquel la combinaison de kinétine et d'acide naphtalène-acétique ou de kinétine et d'acide 2,4-dichlorophénoxyacétique a été ajoutée comme régulateur de croissance des végétaux.

8. Procédé pour induire un cal et faire proliférer des cellules en culture en suspension comprenant les étapes a) à c) de la revendication 1, un gamétophyte femelle de Taxus brevifolia NUTT. ou de Taxus cuspidata SIEB. et ZUCC. étant utilisé comme tissu vivant.

9. Cal ou cellules en culture en suspension obtenu(es) par le procédé selon la revendication 8, contenant au moins du taxol comme alcaloïde.

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(54) **ANTITUMOR COMPOUND NSC-LSC1 AND PRODUCTION THEREOF.**

(57) A taxol analog represented by estimated structural formula (I). It is one of the structural isomers of taxol and intensely inhibits the growth of mouse and human cancer cells. The production process comprises tissue culture of albumens of a plant belonging to the genus *Taxus*.

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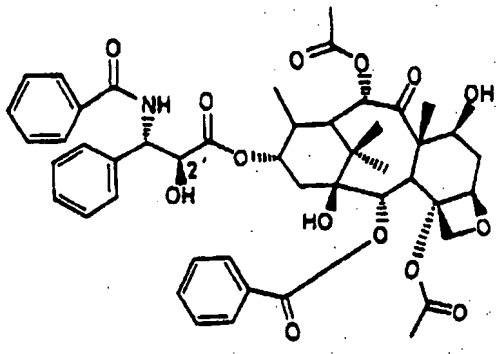


(I)

TECHNICAL FIELD

The present invention relates to NSC-LSC1, which is a novel compound having an antitumor activity belonging to an analogue of taxol, and a process for producing the same. As with the taxol, the novel compound of the present invention has a low toxicity and a high antitumor activity.

BACKGROUND ART

A large number of compounds having an antitumor activity and derived from plants are known in the art, and some of them are actually utilized as an antitumor agent. The utilization thereof has become an important means for treatment of tumors.

In general, many compounds having an antitumor activity and derived from plants are highly toxic to the human body, and also with respect to compounds approved as a drug, the toxicity to the human body is a large obstacle to the use thereof. Under these circumstances, taxol, which is a diterpene of taxane series, has attracted attention as a compound having a low toxicity and a high antitumor activity against human tumors. Taxol, extracted from the bark of Taxus brevifolia NUTT. belonging to the genus Taxus which in turn belongs to the family Taxaceae, has been subjected to clinical tests as an antitumor agent (see, for example, David G.I. Kingston, Pharmac. Ther. Vol. 52, pp. 1-34, 1991). In recent years, the production of taxol by tissue culture of a particular tissue from plants has also been reported (see, for example, U.S. Patent No 5,019,504).

However, the development of a compound having diverse activities would be desired for contribution to chemotherapy useful for diverse malignant tumors, particularly cancers.

Accordingly, an object of the present invention is to develop a novel compound having an antitumor activity, which is expected to have a low toxicity and a specific antitumor activity against human tumors, and to provide a process for producing the same, which enables such a novel compound to be stably supplied.

DISCLOSURE OF THE INVENTION

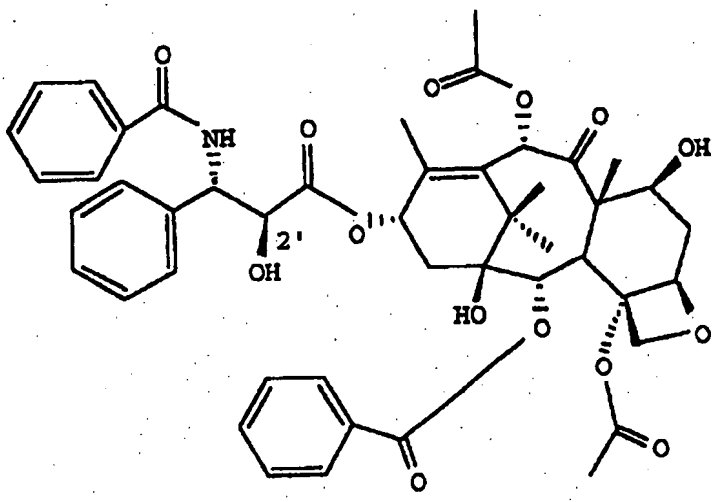
The present inventors have aimed at a compound having an antitumor activity and contained in plants belonging to the family Taxaceae and have made extensive and intensive studies. As a result, the inventors have succeeded in isolating a novel compound from a callus of Taxus brevifolia NUTT., which is clearly distinguished from taxol in the steric structure thereof although it exhibits the same high antitumor activity as that of taxol. Further, it has been confirmed that this compound can be efficiently produced from cali which can be easily induced and proliferated by tissue culture wherein female gametophytes of Taxus brevifolia NUTT is used as an explant. This has enabled the novel compound of the present invention to be stably supplied.

Namely, according to the present invention, there is provided a novel compound having an antitumor activity and identified by the following physicochemical properties:

① form: white powder
② molecular weight: m/e = 854 (MH^+ as measured by FAB mass spectrum (NBA matrix)
③ elementary analysis (found):
carbon 66.1%, hydrogen 6.0%
oxygen 26.3%, nitrogen 1.6%
④ molecular formula: $C_{47}H_{51}NO_{14}$
⑤ specific rotation: $[\alpha]_D^{24}$ = -49.7°
[c = 0.36; methanol]
⑥ melting point: 213 - 216°C
⑦ solubility: sparingly soluble in water; easily soluble in methanol
⑧ proton nuclear magnetic resonance spectrum
(^{1}H-NMR) (400 MHz; deuterated chloroform; chemical shift, ppm; coupling constant, Hz)
1.145 (s, 3H); 1.242 (s, 3H);
1.687 (s, 3H); 1.798 (s, 3H);
1.82 (broad s, 1H); 1.88 (broad s, 1H);
2.238 (s, 3H); 2.35 (m, 2H);
2.385 (s, 3H); 2.55 (m, 2H);]
3.56 (broad, 1H); 3.79 (d, j=6.8, 1H);
4.18 (d, j=7.4, 1H); 4.31 (d, j=8.3, 1H);

4.4 (m, 1H); 4.79 (d, j = 2.4, 1H);
4.95 (dd, j = 1.8 and 7.2, 1H);
5.67 (d, j = 7.3, 1H);
5.79 (dd, j = 2.5 and 8, 1H);
6.23 (t, j = 8.5, 1H); 6.272 (s, 1H);
6.99 (d, j = 8.8, 1H); 7.39 (m, 4H);
7.48 (m, 5H); 7.49 (m, 4H);
7.73 (d, j = 7.5, 1H); 8.13 (d, j = 8.3, 1H).

For example, according to ^{1}H-NMR spectrum among the above-described physicochemical properties, a peak at 3.56 ppm is broad, and the correlation of the peak with a peak at 4.79 ppm is lost, so that is apparent that as represented by the following estimated structural formula, the compound of the present invention is different from taxol at least in the bonding form of a hydroxyl group at the 2'-position and is therefore structurally isomeric with taxol.



The present inventors have designated this compound as an antitumor compound NSC-LSC1 (hereinafter abbreviated "NSC-LSC1").

Further, according to another aspect of the present invention, there is provided a process for producing NSC-LSC1, which comprises steps of culturing a callus derived from female gametophytes of Taxus brevifolia NUTT., and extracting NSC-LSC1 from the resultant cultured product.

BEST MODE FOR CARRYING OUT THE INVENTION

The estimated structural formula of NSC-LSC1 specified by the above-described physicochemical properties is supported also by the following ^{13}C nuclear magnetic resonance spectrum (^{13}C-NMR).

^{13}C-NMR (400 MHz, deuterated chloroform, chemical shift, ppm):

9.58	72.2	167.11
14.85	72.43	167.77
20.85	73.21	170.38
21.84	74.97	171.27
22.63	75.59	172.76
26.89	76.54	203.65
35.63	77.63	
35.72	79.08	
43.22	81.19	
45.65	84.43	
55.08	125-135	
58.65	131.25	

NSC-LSC1 provided by the present invention exhibits a high proliferation inhibitory activity against human or animal cancer cells transplanted into a test animal and further, a low toxicity to the animal which renders NSC-LSC1 promising as a compound for use in the field of chemotherapy of cancers.

The results of tests carried out at Institute of Applied Microbiology in the University of Tokyo, on proliferation inhibitory activity (IC_{50}) against P388 (mouse leukemia) and KB cells (human squamous cell carcinoma) are provided in Table 1. The IC_{50} value is expressed in terms of the concentration of a drug required to reduce the cell proliferation by 50% to a control group (for reference, it is noted that in the antitumor effect evaluation test carried out at the National Cancer Institute (NCI) of the National Institute of Health in the U.S.A., substances having an IC_{50} value against KB cells of 4000 ng/ml or less for synthetic substances and 20000 ng/ml or less for naturally occurring substances are defined as an effective antitumor substance.)

Table

Inhibitory Activity of NSC-LSC1 against Proliferation of Mouse and Human Cancer Cells	
Test Cells	IC_{50} (ng/ml)
• P388	3.3
• KB cells	19

As is apparent from the above-described results, NSC-LSC1 exhibits a very high antitumor activity against the human tumor cells.

NSC-LSC1 of the present invention can be advantageously produced by the process of the present invention which will now be described.

Specifically, according to the present invention, there is provided a process for producing a compound NSC-LSC1 by culturing tissues originating from Taxus brevifolia NUTT., which comprises:

a) a step of preparing an explant from living female gametophytes;

b) culturing the tissue prepared in the step (a) in a nutrient medium suitable for the induction of a callus to induce a callus;

c) culturing the callus obtained in the step (b) in a nutrient medium suitable for the proliferation of the callus; and

d) harvesting NSC-LSC1 from the resultant cultured product in the step c).

Examples of the preparation of an explant from living female gametophytes involve steps in which seeds collected from Taxus brevifolia NUTT. are sterilized with 70% ethanol and an aqueous solution of sodium hypochlorite, and the seeds are then aseptically cut open to remove female gametophytes, and thereafter, the female gametophytes are planted on a medium solidified by agar or Gelrite® (gellan gum manufactured by Merck & Co., Inc.) suitable for inducing a callus as described hereinbelow.

The living tissues obtained in the above-described steps are then cultured on a nutrient medium suitable for the induction of a callus and the induced callus is cultured in a nutrient medium suitable for the proliferation of callus cells. The primary components to be used in the nutrient medium include water; mineral nutrients, e.g., nitrogen (ammonium salts and nitrates), phosphorus, potassium, calcium, magnesium, sulfur, etc.; sugars, e.g., sucrose, glucose, fructose, maltose, etc.; minor amounts of inorganic nutrients; organic substances such as vitamins and amino acids; naturally originating substances such as

coconut milk; and optionally, agar, Gelrite®, alginic acid, and agarose. The basic formulations of mineral nutrients used as basic media include Schenk & Hildebrandt medium (hereinafter referred to "SH medium"), Murashige & Skoog medium (hereinafter referred to as "MS medium"), Gamborg's B5 medium, White's medium, Nitsch & Nitsch medium, Nagata & Takebe medium and Woody Plant medium. To these basic media are further optionally added, as plant growth regulators, auxins, for example, 1-naphthaleneacetic acid (hereinafter referred to as "NAA"), indole-3-acetic acid (hereinafter referred to as "IAA"), indole-3-butyric acid (hereinafter referred to as "IBA"), and 2,4-dichlorophenoxyacetic acid (hereinafter referred to as "2,4-D"; cytokinins, for example, benzyladenine (hereinafter referred to as "BA"), kinetin, zeatin, and 6-furfurylaminopurine; and gibberellins (hereinafter referred to as "GA", for example, GA_1 and GA_3). Media comprising the above described basic media and, added thereto, auxins, cytokinins and gibberellins are preferable as the nutrient media suitable for inducing the callus according to the present invention. More specifically, a medium comprising the SH medium as the basic medium and, added thereto, BA as the cytokinin and GA_3 as the gibberellin, or a medium comprising the SH medium as the basic medium and added thereto, NAA as the auxin and kinetin as the cytokinin is preferable. On the other hand, the nutrient media suitable for proliferating the callus cells preferably comprises a medium comprised of the above-described basic medium and, added thereto, cytokinins and auxins, more specifically a medium comprising the SH medium as the basic medium and, added thereto, kinetin as the cytokinin and NAA or 2,4-D as the auxin. The pH level of such media is adjusted to 5 to 7, preferably 5.5 to 6.0 with an appropriate acid or alkali.

The above-described cultures using these media can be carried out at a temperature in the range of 15-25 °C with or without light-irradiation. In particular, the cultures for the proliferation of the callus cells are preferably carried out in a liquid medium by making use of a shaker.

The isolation of NSC-LSC1 from the cultured products thus obtained can be carried out according to the method of isolating various alkaloids from cultured tissue followed by purification, which is known per se. The term "cultured products" used herein is intended to include cultured cells and clumps (cali) of cells and their suspensions particularly when use is made of liquid media. Examples of the method of extracting NSC-LSC1 from the cultured product includes, but is not limited to, a general method which comprises separating cali from media, drying and pulverizing the same, subjecting the resulting powder to extraction with an appropriate organic solvent, optionally washing the organic phase with water, drying the organic phase, and then removing the solvent by distillation. If necessary, the extracted NSC-LSC1 can be purified by various chromatographic treatments or recrystallization, etc. Examples of the organic solvent which can be used in the extraction include chlorinated hydrocarbons such as methylene chloride and dichloroethane, alcohols such as methanol, ethanol and isopropyl alcohol, and mixtures of the above organic solvents.

The present invention will now be described in greater detail by referring to the following working examples, to which the present invention is not restricted.

EXAMPLE

A seed of Taxus brevifolia NUTT. (a native tree which grows at Fish Lake, Oregon, U.S.A.) from which aril had been removed and stored in a refigerator was immersed in an aqueous solution of 70% W/W ethanol for one minute, and then in an aqueous sodium hypochlorite solution having an effective chlorine concentration of 1% for 20 minutes to sterilize the seed, and thereafter, was rinsed three times with sterilized water. After the sterilization, the hard external seed coat and internal seed coat were removed with a knife and tweezers, and the resulting embryos and female gametophytes were then separated from each other.

The female gametophytes thus prepared were planted on a SH medium (supplemented with 0.25% of Gelrite to provide a solid medium) prepared by adding two plant growth regulators, benzyladenine (BA) and GA3, which is a kind of gibberellin, and adjusting the pH value to 5.8. The concentrations of BA and GA3 were 1, 5 or 10 mg/l and 0, 1, 10 or 100 mg/l, respectively. The planted female gametophytes were cultured at a temperature of 20 °C or 25 °C under a light (for 16 hr per day). As a result, it was found that a callus was induced from the gametophytes after approximately 2 months of the initiation of the culture.

The callus thus induced was subcultured in a SH medium (a liquid medium) supplemented with naphthaleneacetic acid (NAA) and kinetin and by adjusting the pH value to 5.8. The concentrations of NAA and kinetin added to the medium were 5 mg/l and 0, 0.1 or 0.5 mg/l, respectively. The amount of the medium was 40 ml per Erlenmyer flask having a capacity of 100 ml, and suspension culture was conducted at 100 rpm by using a flask shaker. The culture was conducted at a temperature of 20 °C under a lighting condition darkness.

Four weeks after the initiation of the culture, the proliferation of the callus was examined. As a result, with respect to the medium to which NAA alone had been added to a concentration of 5 mg/l, the proliferation rate was about 5 times that of the callus planted in the above-described liquid medium. Further, with respect to the medium to which NAA alone had been added to a concentration of 5 mg/l, the culture was conducted at a temperature of 20 °C or 25 °C and under darkness or illumination (for 16 hr per day) for 4 weeks. As a result, proliferation was observed in every combination, however, the best result was obtained particularly when the culture was conducted at 20 °C in darkness. Further, when 2,4-D was used instead of NAA as the auxins, proliferation similar to that in the case where use was made of NAA was observed.

The callus thus proliferated was harvested and dried. The dried callus of 30g was throughly homogenized in a mortar, combined with 500 ml of a mixed solution comprising methanol and methylene chloride in a ratio of 1 : 1, and shaking and extraction were conducted at room temperature for 18 hr in a Sakaguchi flask. This procedure was repeated three times, and the extract was concentrated in vacuo to provide 1.03g of a brown substance. The brown substance was subjected to partitioning with 500 ml of a mixed solution comprising methylene chloride and water in a ratio of 1 : 1 to recover the methylene chloride layer. The above procedure was repeated several times, and the extract was concentrated in vacuo to provide 530 mg of a brown substance. The resultant compound was dissolved in methanol and fractionated by high performance liquid chromatography [column: Dynamax (trademark), 60A, 8 μm, C18, 4.6 mm$^{\phi}$ 250 mm (manufactured by Rainin Instrument Company Inc., U.S.A.); composition of mobile phase: comprising methanol, water and acetonitrile in a ratio of 20 : 41 : 39].

Among the resultant fractions, a fraction considered to have activity was concentrated in vacuo. This procedure was repeated to provide 11 mg of a white powdery substance. This powder was dissolved in methanol and subjected to high performance liquid chromatography [column: Dynamax (trademark), 60A, 8 μm, C18-phenyl 4.6 mm$^{\phi}$ 250 mm (manufactured by Rainin Instrument Company Inc., U.S.A.); composition of mobile phase: comprising methanol, water and acetonitrile in a ratio of 20 : 45 : 35; detecting light wavelength: 227 nm] to determine the absorption of the detecting light wavelength to the retention time of the column. As a result, only one peak was observed, that is, it was found that a compound having a very high purity was provided.

The high-purity compound was found to have the above-described physicochemical properties and antitumor activity.

INDUSTRIAL APPLICABILITY

The NSC-LSC1 of the present invention has a low toxicity and a high antitumor activity and can be stably supplied by tissue culture wherein use is made of female gametophytes of Taxus brevifolia NUTT., which renders the present invention utilizable in the manufacture of pharmaceuticals.

Claims

1. A compound NSC-LSC1 analogous to taxol and identified by the following physicochemical properties:
   (i) form: white powder
   (ii) molecular weight: m/e = 854 (MH^+) as measured by FAB mass spectrum (NBA matrix)
   (iii) elementary analysis (found):
   carbon 66.1%, hydrogen 6.0%
   oxygen 26.3%, nitrogen 1.6%
   (iv) molecular formula: $C_{47}H_{51}NO_{14}$
   (v) specific rotation: $[\alpha]_D^{24}$ = -49.7°
   (c = 0.38; methanol)
   (vi) melting point: 213 - 216 °C
   (vii) solubility: sparingly soluble in water; easily soluble in methanol
   (viii) proton nuclear magnetic resonance spectrum
   (^{1}H-NMR) (400 MHz; $CDCl_3$, ppm)
   1.145 (s, 3H); 1.242 (s, 3H);
   1.687 (s, 3H); 1.798 (s, 3H);
   1.82 (broad s, 1H); 1.88 (broad s, 1H);
   2.238 (s, 3H); 2.35 (m, 2H);
   2.385 (s, 3H); 2.55 (m, 2H);
   3.66 (broad, 1H); 3.79 (d, J=6.8, 1H);
   4.18 (d, J=7.4, 1H); 4.31 (d, J=8.3, 1H);

4.4 (m, 1H); 4.79 (d, j = 2.4, 1H);
4.95 (dd, j = 1.8 and 7.2, 1H);
5.67 (d, j = 7.3, 1H);
5.79 (dd, j = 2.5 and 8, 1H);
6.23 (t, j = 8.5, 1H); 6.272 (s, 1H);
6.99 (d, j = 8.8, 1H); 7.39 (m, 4H);
7.48 (m, 5H); 7.49 (m, 4H);
7.73 (d, j = 7.5, 1H); 8.13 (d, j = 8.3, 1H).

2. A process for producing a compound NSC-LSC1 according to claim 1 by culturing tissues originating from Taxus brevifolia NUTT, which comprises:
a) a step of preparing an explant from living female gametophytes;
b) culturing the tissue prepared in the step (a) in a nutrient medium suitable for the induction of a callus;
c) culturing the callus obtained in the step (b) in a nutrient medium suitable for the proliferation of the callus; and
d) harvesting NSC-LSC1 from the resultant cultured product in the step c).

INTERNATIONAL SEARCH REPORT

International Application No PCT/JP92/00917

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl[5] C07D305/14, C12P17/02//A61K35/78, A61K31/335

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C07D305/14, C12P17/02, A61K35/78

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US, A, 5019504 (US sec of Agriculture), May 28, 1991 (28. 05. 91), (Family: none)	2
A	Journal of Natural Products, Vol. 49, No. 4, p. 665-9 (1986), C. H. Oliver Huang, D. G. I. Kingston et al. "New Taxanes from Taxus Breuifolia, 2"	1
A	Journal of Organic Chemistry, Vol. 54, No. 8, p. 1805-10 (1989), G. L. Gordon et al., "Ring expansion of [2+2] Photoaducts"	1

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
October 6, 1992 (06. 10. 92)	October 27, 1992 (27. 10. 92)
International Searching Authority	**Signature of Authorized Officer**
Japanese Patent Office	